ENSILING PROCESS I. GREEN MAIZE ENSILAGE AND ITS MICROBIAL RESEARCH

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By

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In the Tôhoku District, mainly from the economical point of view, a simple silo is widely used for the preparation of winter feedings. It is a very small-scaled tower measuring 1.5 m. in diametre and 3.0 m. in depth, and made of concrete, or even merely a raugh hole dug in the earth. The ensilage preparation in such a simple silo is so perishable that there is an increasing yearly loss in animal feedings.

To make clear the putrefaction process of the ensilage in such a silos might lead to a safety, ease and economic method for its preparation. For this purpose, the following experiments have been caried out. The green maize ensilage was prepared in the earth-pit silos, and the raise and fall of the constituents in its ensiling process as well as microbial actions were investigated. Many strains of aerobic micro-organisms were isolated from this ensilage, especially from the putrefied part. When classified into groups by their physiological properties, some strains of them were recognized as well acid-tolerable, so that they were assumed to cause the ensilage putrefaction. All these strains belong to the yeast, which had high metabolic activities against the acid so far examined, as well as growing ability on the plain broth, though not so well.

Materials and Methods

A) Raise and fall of the constituents in the ensiling process and action of several micro-organisms in the groups.

I) *The Ensilage Preparation.*

Green maize, which was harvested at the Kawatabi Farm on 30th August, was used as the ensiling material. The fresh materials (harvested one day before packing) were chopped and packed into the earth-pit silo (1 m. in diametre

and 2 m. in depth). Then, the weights of 200–300 kg. were placed upon them. The ensiled materials of pH 5.2 have in average, 78.8 % moisture, 2.68 % water-soluble sugar, 0.56 % water-soluble nitrogen, 0.62 % non-volatile acid as lactic acid, and 0.68 % volatile acid as acetic acid.

II) *Sampling and Analysis.*

The samples for analysis were collected from each silo on regular day-interval after the packing; they were taken from 4 portions of the ensiled pile, namely at 5–10 cm. depth from the surface, 100–120 cm. depth in the center, 100–120 cm. depth at the distance of 5–10 cm. from the wall, and at 5–10cm. from the bottom.

The samples were crushed through a mincer, then placed in a jar which was filled 10 times its volume with distilled water. Suspensions thus obatined, were shaken for 30 min. and then used for analysis. Acidity (total acid, volatile acid and non-volatile acid) was estimated by titration with N/10 NaOH. The filtrate of the suspension were analysed of the water soluble sugar and nitrogen, the former by Bertrand's method and the latter by Kjeldahl's method respectively.

The microbes in the samples were assumed provisionally to be grouped into acid-former, aerobes, and anaerobes. Each group was detected by cultivation in Koji-juice-$CaCO_3$-, broth- and glucose-broth-agar medium respectively, whereupon the number of the colonies that emerged, was also counted. Anaerobic condition was made by agar-layer method.

B) Classification of the microbes by their physilogical properties.

To classify the microbes by the physiological properties of 80 isolates, which grew preliminarily on the plain broth, the growing test on the following 10 kinds of media was carried out.

Medium (1) 1% glucose+0.1% ammonium sulfate,
,, (2) 1% glucose+0.2% carbon treated pepton,
,, (3) 1% glucose+0.2% pepton in vegetable extract,
,, (4) 1% glucose+0.2% pepton in yeast extract,
,, (5)~(8) 1% solium lactate was used instead of 1% glucose, corresponding to media (1)~(4),
,, (9) 1% pepton water,
,, (10) 1% carbon treated pepton water.

Each medium was adjusted to pH 7.2.

Among the glucose containing media (1)–(4), assimilability of inorganic nitrogen was tested in medium (1), whilst other media were used for the test on the assimilation of organic nitrogen in the presence of growth factors from various origins, which are supplemented either sufficiently, or insufficiently. In the media (5)–(8), glucose was replaced with lactate as a carbon source. Media

(9)–(11) were used for the test on the demand of growth factors in the absence of the carbon source.

C) The metabolic activities of the yeast strains against the organic acids.

The metabolic activities against the acids were reserached with 5 yeast starins out of 80 isolates, according to their acid-tolerance. Analogous to the naturally occurring carbon sources with which the yeasts had encountered in the ensiling process, the following 2 media were employed for precultivation.

Medium I ; 1.0% glucose, 0.5% broth, 0.5% pepton, and 0.25% NaCl.

Medium II ; with 1.0% Na-lactate replaced glucose.

Medium I, composed of glucose as the carbon source, corresponded to the circumstances in the initial stage of the ensiling process, whilst medium II, which contained lactate instead of glucose, to those in the ensiling stage, when the sugars in the ensilage were exhaustively consumed and the acid became a sole source of carbon for microbial reproduction. The yeasts were cultured at pH 6.3 for 40 hours in each medium, harvested and then used for research on its metabolic activity against the acids added. The activity was measured manometrically at 37° in Warburg's conical vessels, in which 2.0 ml. of HCl-phosphate buffer (pH 3.5), 1 ml. of yeast suspension, 0.5 ml. substrate solution, and 0.3 ml. of KOH were contained, unless otherwise cited. The temperature as well as pH at the time of measurement corresponded to those in the equilibrium stage of the ensiling process. M/5 glucose, M/5 Na-lactate, M/5 lactic acid, M/5 acetic acid and M/5 butyric acid were used as substrate respectively.

No. 32 (film-former) and No. 39 (non-film-former) were investigated as test strains, and Sake yeast Kyôkai No. 6 was used as a control.

Results and Discussion

A) Raise and fall of the constituents during the ensiling process and action of micro-organisms in the groups.

According to the changes in the chemical constituents and number of the microbes, the ensiling process can be distinguished into 4 phases. (see Fig. 1, 2, 3 and 4)

(1) *Phase I.* This phase is the duration from the 1st to the 3rd day after packing of the materials into silo. It is characterized by the production of non-volatile acid with the consumption of reducing sugar and also by the decrease of volatile acid at every portion of the ensiled pile. Since the former acid largely exceeds the latter one in quantities, the accumulation of the total acid reaches its maximum throughout the process especially at the center and the marginal portion (designated the "side"), and accordingly, it takes values of 2.5% and 2.0% respectively. The hydrogen ion concentration is pH 4.0 at the center, and pH 4.2 at the side and bottom.

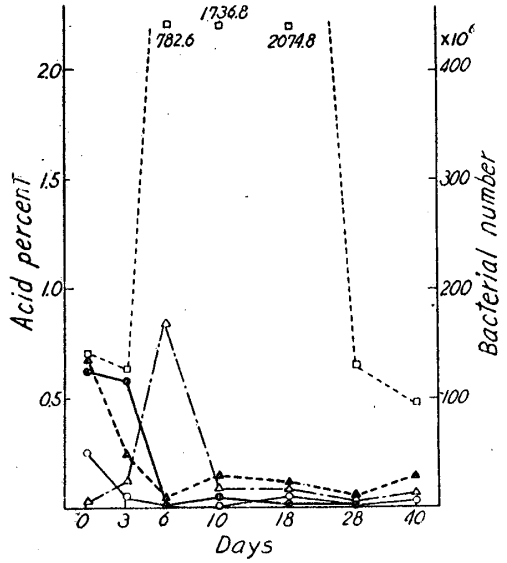


Fig. 1. Changes in the acids and organisms in the ensiling process *at the Surface portion.*

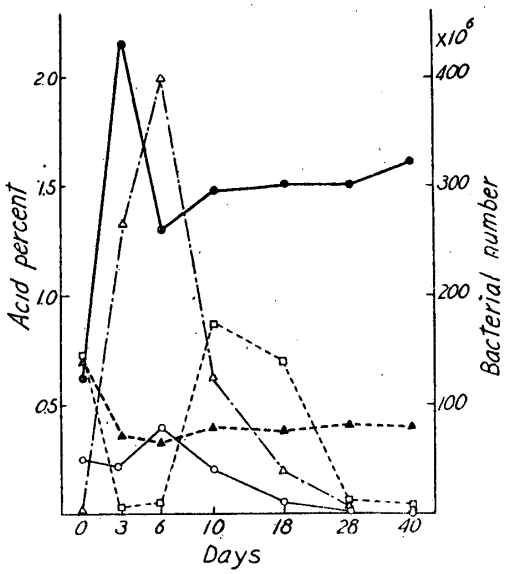


Fig. 2. Changes in the acids and organisms in the ensiling process *at the Center portion.*

(●——●) non-volatile acid ; (▲·····▲) volatile acid ;
(○——○) acid forming organisms ; (□·····□) aerobic organisms ;
(△ – – –△) anaerobic organisms.

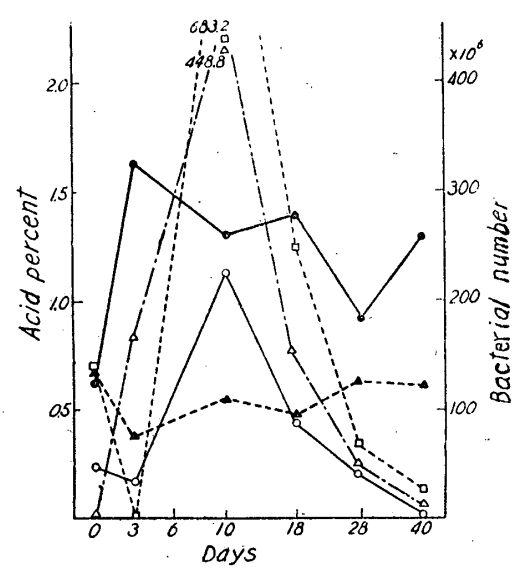


Fig. 3: Changes in the acids and organisms in the ensiling process *at the Side portion.*

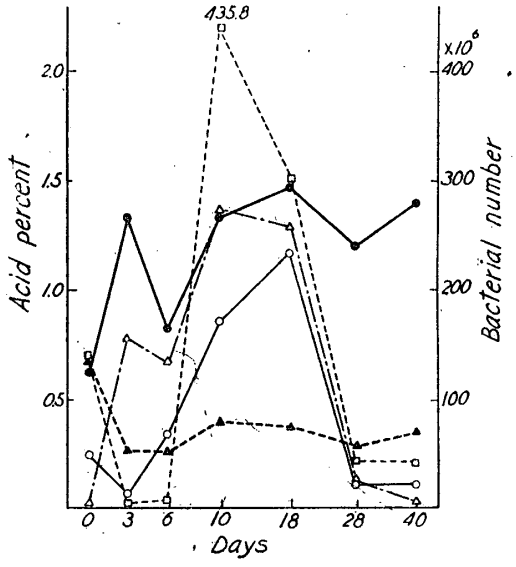


Fig. 4. Changes in the acids and organisms in the ensiling process *at the Bottom portion.*

(●——●) non-volatile acid ; (▲·····▲) volatile acid ;
(○——○) acid forming organisms ; (□·····□) aerobic organisms ;
(△ – – –△) anaerobic organisms.

In every portion exclusive of the surface the aerobes decrease rapidly, while, on the contrary, the anaerobes increase. At the surface, where it is aerobic, the interchange of the aerobes with anaerobes is moderate ; this indicates that every portion of the ensiled pile other than the surface become fairly anaerobic.

In spite of the accumulation of the acid the counts of the acid-formers were observed to be fairly small and rather inclined to decrease. Thus it is necessary to advance studies on the detailed composition of micro-organisms grouped in the acid-formers and the anaerobes.

Water soluble reducing sugar are consumed upto 0.15–0.18% in 3 days at every portion.

(2) *Phase II.* This phase includes the period of a week following that of phase I. Whilst both the water soluble nitrogen and sugar show no remarkable changes in their quantities, the non-volatile acid accumulated once during the the phase I decreases at first and then re-increases in this phase. The changes in the volatile acid follow almost the same course as those in the non-volatile one, though the quantity is small. These changes in acid accumulation are observed most distinctly at the center, and also considerably at the side and the bottom respectively. Thus, this phase can be chracterized as the period of fluctuation in the acid accumulation. In this period the total amount of acid is inferior to that of phase I, but the pH value gives the lowest value throughout the ensiling process in every portion. Especially the value of pH at the center is so low as 3.4, such an acid circumstance, associating with anaerobic conditions, gives an inhibitory influence upon the growth of the putrefactive aerobes.

The maximal number of the acid forming bacteria so far counted, differed with the portion of the ensiled pile, i.e. on the 6th day it was at the center, on the 10th day at the side, and on the 18th day at the bottom : thenafter their number inclined to decrease rapidly. The anaerobes varies also in numbers about equally with the acid-formers. The retortion of the day, when both organisms reach their maximal count, according to the place of the portion, corresponds to the state of the aerobiosis govering, each portion.

This is also the case with the aerobic baceria, whose number began to increase from the 6th day, and soon reached its maximum in each portion by the 10th day.

At the surface, where it is most aerobic, the volatile as well as non-volatile acid decreased continuously with no re-increase, until at the end of this period the amount of the former acid was lowered as far as 0.15% and the later to 0.05%. Despite this decrease in acid, the value of pH at this portion remained at 4.5 till the 15th day, whereupon the areobes burst into multiplication so vigorously, that they gained the overwhelming majority soonafter. This is rather curious, because it is generally assumed that such aerobes are not able to grow well in acid pH. Thus, there might be some precursor, which attacks the acid in a way

to promote good growth of the aerobes. Actually some yeast strains, which are capable to attack the acid, such as lactic or acetic acid, were isolated from the side. It is assumed that the acid that accumulated was consumed by these active yeast strains to influence the bacterial growth, as above mentioned.

(3) *Phase III.* This phase includes the period of a month successive to that of phase II, and is the phase of equilibrium in the acids. A typical statue of this stage is well established at the center, where the amount of the volatile and non-volatile acids are left unvaried, and also where the pH-value is well maintained at 3.6 to 3.8 during this long period. At this portion almost all the aerobes cease to play any active role due to the low hydrogen ion concentration as stated above, including the deficiency in the nutrient under anaerobic conditions. At the somewhat more aerobic portion, e.g. at the side or at the bottom, some fluctuation in acids like the character of the phase II are observed, and as a rule there is a tendency to decrease. Thereupon, the viable number of the aerobes at both of these portions remains larger than at the center. This might be a probable cause of such acids-fluctuation as above observed.

(4) *Phase IV.* This phase is the last stage of the ensiling process. In this stage, the range of the spoilage at all the portions which has been suffering from it, is further enlarged due to the depression of the acid present in them with the elapse of time. For example, where a putrefactive symptom was discernible a little on the 10th day, it was rather distinctly observed on the 100th day, whereupon the non-volatile acid was analysed as 0.24% and the volatile one as 0.18%. This might be well mentioned to be the case also at the bottom. At the surface, which might be rather characteristic in its putrefactive tendency due to its close constant with the air, the putrefaction proceeds on its course just from the first step, so that the acid contained in it was already depressed considerably on the 3rd day, and its pH-value reached 8.0 on the 28th day.

In this phase the aerobes, which had acted violently in the preliminary phases, fall down into decay even at the surface, where only a small number of them remained alive.

Since the samples were taken from the four limited portions of the ensiled pile in each different silo according to the sampling day and then analysed respectively, it is reasonably accepted that there may be some fluctuations in the data thus obtained. The rough concept on the groups of microbes simply as aerobes, anaerobes and acid-formers are quite conventional, and may not probably reflect the true statue of the microbial state. Nevertheless, the four phases as above cited, are well distinguishable in the ensiling process. These phases are converted stepwise due to the microbial actions, which, on the other hand, are governed by the factors in circumstances, either established by themselves or not.

One of the main factors governing almost a whole part of the ensiled pile other than its surface portion throughout the process is a considerably acid milieu, in which some bacterial growth, especially of the putrefactive aerobes is affected inhibitorily. With this respect, these might be considered the role of acid-forming organisms, or of acid tolerable ones in the ensiling process. General concept on the acid-forming organism in the ensiling proces has concern mainly with Lactobacillus. However, this is not well fittable to elucidate the contradictional result between acid accumulation in high amounts on the one hand and the decrease in the numbers of the acid-formers on the other hand, as seen especially in phase I. In this case it would be more reasonably to mention that the concerned acid-forming organisms may be anaerobes or yeasts. The parallelism between acid accumulation and number of acid formers was observed only in the phase II, at the bottom portion, where the aerobiosis was concerned to a much less extent. In such a case Lactobacillus might be assumed as the main cause for acid accumulation.

The other governing factor, though partly, in portions, is the aerobiosis. It is very interesting to note that in the portions which are concerned with the aerobiosis to a less extent, the growth of the anaerobes is rather proportional to that of the aerobes, though in limiting ranges, as seen at the side or at the bottom of the ensiled pile respectively, and seems to display a protective role from the putrefaction. The correlation between the growth of both organism will be further studied in the future.

At any rate, it is obvious that the portion governed by the aerobiosis in the ensiled pile falls soon or later into the putrefaction. The event of the purtefaction occurs even at such acid pH as untolerable for the putrefactive aerobes. This might lead to the assumption on the concerned organisms as acid-tolerable and actively metabolic against free acids, not their salts, under aerobic conditons.

B) Classification of the organisms grown aerobically on the nutrient broth due to their physiological properties.

Among the aerobes, which were occasionally isolated at random from colonies grown on the broth-agar medium by inoculation of the samples during the ensiling process, 80 strains were selected for classification according to their physiological properties. These 80 strains were divided into sixteen groups by their demand of the carbon sources, organic nitrogen sources, and several vitamins from various origins. These results are summarized in Table 1.

These strains were also studied as to their tolerance against the high hydrogen ion concentration. This was done by the use of acidified media added with lactic acid in three grades, ranging from 0.5% to 1.5%. pH of the medium containing 1.5% lactic acid was noted as 2.4 As shown in Table 2, five strains, well tolerable to this medium were obtained ; then are Nos. 30 31, 39, 32 and 58.

Table 1. The classifications of the bacteria grown in nutrient-broth due to its physiological characters.

Group I Will need a carbon source other than pepton (Does not grow in pepton water).
Strain number. 14, 17, 18, 27, 30, 31, 34, 36, 39, 42, 45, 52, 53, 54, 59, 63, 68, 70, 72, 74, 75, 77, 79, 80, 85, 86, 88, 89, 101 (30 strains).

Ia Will utilize lactate (Will grow in lactate-pepton water).
14, 18, 30, 31, 36, 39, 42, 45, 52, 54, 59, 68, 75, 85, 88, 101 (16 strains).

Iaa Will utilize NH_3-N as nitrogen source (Will grow in lactate-NH_3-medium).
88 (1 strain).

Iab Does not utilize NH_3-N, and needs organic nitrogen (Will grow in lactate-pepton water instead of lactate-NH_3-medium).
14, 30, 31, 36, 39, 42, 45,52, 54, 59, 68, 75, 85, 88, 101 (15 strains).

Iaba Will need the vitamins.
36, 75, 85, 101 (4 strains).

Iabaa Will need the vitamins from vegetable.
36 (1 strain).

Iabab Will need the vitamins from yeast and pepton (Will grow in lactate-yeast extract and -pepton instead of vegetable extract).
36, 75, 85, 101 (4 strains).

Iabb Does not need the vitamins (Will grow in a vitamin deficient medium such as lactate-carbon-treated-pepton water).
14, 18, 30, 31, 39, 42, 45, 52, 54, 59, 68 (11 strains).

Ib Does not utilize the lactate (Will grow in glucose-pepton water instead of lactate-pepton water).
17, 27, 53, 63, 70, 72, 79, 80, 89, 95 (10 strains).

Iba Will utilize NH_3-N as nitrogen source.
17 (1 strain).

Ibb Does not utilize NH_3-N, and will need organic nitrogen.
27, 53, 72, 79 (4 strains).

Ibba Will need the vitamins.
27, 53, 72, 79 (4 strains).

Ibbaa Will need the vitamins from vegetable.
72, 79 (2 strains).

Ibbab Will need the vitamins from yeast or pepton.
27, 53 (2 strains).

Group II Does not need a carbon source other than pepton (Will grow in pepton water).
1, 3, 5, 6, 8, 9, 10, 22, 22-1, 23, 24, 25, 26, 28, 29, 32, 35, 37, 38, 41, 43, 44, 46, 47, 48, 49, 50, 51, 57, 58, 61, 62, 64, 65, 66, 67, 71, 73, 76, 78, 83, 87, 90, 91, 92, 94, 97, 99, 102 (50 strains).

IIa Will utilize lactate (Will grow in lactate-pepton water).
22, 23, 24, 26, 28, 29, 32, 38, 41, 47, 48, 49, 57, 58, 61, 62, 64, 67, 71, 78, 91, 97, 99, 102 (24 strains).

IIaa Will utilize NH_3-N.
38, 48, 57, 29, (4 strains)

IIab Does not utilize NH_3-N, and will need a organic nitrogen.
22, 23, 24, 26, 28, 33, 41, 47, 49, 58, 61, 62, 64, 67, 71, 78, 91, 97, 99, 102, (20 strains).

IIaba Will need the vitamins.
23, 26, 29, 49, 62, 64, 67, 78, 99, 102 (10 strains).

IIabaa Will need the vitamins from vegetable.
23, 26, 29, 62, 67, 78 (6 strains).

IIabab Will need the vitamins from yeast and pepton.
49, 64, 99, 102 (4 strains).
IIabb Does not need the vitamins, and will need the amino acid.
22, 24, 28, 32, 41, 47, 59, 61, 71, 91, 97 (10 strain), and (49, 64)*
IIb Unable to utilize the lactate.
1, 3, 5, 6, 8, 9, 10, 22-1, 25, 35, 37, 43, 44, 46, 50, 51, 65, 66, 69, 73, 76, 83, 87, 90, 92, 94 (24 strains).
IIba Will utilize NH_3-N as nitrogen sources.
1, 35, 43, 44, 46, 50, 66, 73, 83, 92, 94 (11 strains).
IIbb Does not utilize NH_3-N.
3, 5, 6, 8, 9, 10, 22-1, 25, 37, 51, 65, 69, 73, 76, 83, 87, 90 (17 strains).
IIbba Will need the vitamins.**
(73, 83) 51, 65, 90 (5 strains).
IIbbaa Will need the vitamins from vegetable. (0)
IIbbab Will need the vitamins from yeast and pepton.
51, 65, 73, 83, 90 (5 strains).
IIbbb Does not need the vitamins.
3, 5, 6, 8, 9, 10, 22-1, 25, 37, 69, 76, 87 (12 strains).

* Nos. 49 and 64 grow in lactate-pepton water and vitamins free pepton water, but not in lactate-vitamin-free-pepton water. The lactate shows inhibitory effect on the growth of these two strains when the vitamins are absent, while it does not when they are present. No. 29 can not utilize the organic nitrogen when the vitamins or the lactate are given.

** Nos. 65 and 90 do not grow in the glucose-pepton water, and growth of the No. 51 is checked by the lactate. Nos. 73 and 83 do not assimilate organic nitrogen when the vitamins are not supplied, even though it can grow in inorganic nitrogen and glucose medium, thus it requires the vitamins for the assimilation.

The strains which require the vitamins from vegetable can utilize the vitamins from yeast and pepton, while the reverse is not the case.

These strains (Nos. 34, 74, 77) show scanty growth in any culture.

Strains Nos. 30, 31 and 32 were isolated from the side portion on the 16th day, while Nos. 39 and 58 from the same portion on the 10th day, or on the 18th day respectively. These five strains are distinguishable not only by their high acid-tolerance, but also by their unnecessity for special vitamins for their growth, since they were able to grow on the carbon-treated pepton water added with lactate. Among them, the former three strains Nos. 30, 31, 39 differed from

Tabl 2. Growth of the organisms on the medium acidfied with lactic acid*.

Lactic acid %	0.5	1.0	1.5
pH / Strain	3.8	2.8	2.4
22	±	±	
30	++	+	+
31	++	++	++
32	+++	+++	+++
39	++	++	++
41	±	±	
58	+++	+++	+++
67	±	±	±
68	±	±	±
78	±		
85	±		
91	±	±	±
97	±	±	
102	±	±	

* Strains other than in the table showed no discernible growth in every medium tested.

Nos. 32 and 58, with respect to their requirement of the carbon sources. Further difference is also observed in their shape the former are shaped oval to elliptical, while the latter are elliptical to sausage-like. All these five strains are assumed to belong to the yeast by their shape and physiological properties. Thus, the yeast might be mentioned as one of the most acid-tolerable organisms in the ensilage microbes.

C) The metabolic activities of the yeast starins against the acids.

The acid-tolerable strains above obtained were studied in more detail as to their respiratory and fermentative activities against free acids such as free lactic or acetic acid. Two strains Nos. 32 (film-former) and 39 (non-film-former) are used as the representatives of the acid tolerable yeasts, while the Kyôkai No. 6, one of the most typical strains of the Sake-yeast, as a control. Two kinds of media (see p. 389) for cultivation of the yeast were prepared to detect the effects of subculture on the metabolic activities against the acids and glucose, which were researched in acid milieu at pH 3.5.

Table 3. Oxygen uptake against the acids and glcose.

Substrate	Lactic acid $-Qo_2$		Acetic acid $-Qo_2$		Sodium lactate $-Qo_2$		Glucose $-Qo_2$	
Strain \ Medium	I	II	I	II	I	II	I	II
No. 32	37	44	41	66	33	62	48	55
No. 39	33	140	34	23	26	—	45	96
Kyôkai No. 6	18	—	18	—	25	—	22	—

O_2 uptake against the acids and glucose measured at 37°, and pH 3.5 (phosphate). Organisms have been cultured at 30° for 42 hours in medium I or II with shaking. Molarity of each substrate solution is M/35.

The results on the respiratory activities of the organisms thus obtained are shown in Table 3, Cells harvested from medium I show almost the same tendency in oxygen uptake against every substrate indifferently with the strains, whereas in quantities of oxygen absorbed against other substrates than sodium lactate the test strains Nos. 32 and 39 exceed about twoholds that of the Sake-yeast. In this case Qo_2 of each strain is considerably larger than its Qo_2 against other substrates. This means that the organisms, if cultured in the presence of glucose, is able to metabolyze the glucose more actively than free acetic or lactic acid. In the case, where the mdium II is used, the strain No. 32 shows higher activities against acetic acid and sodium lactate, but somewhat lower ones against lactic acid than against glucose, whereupon the strain No. 39 shows remarkably higher activities against lactic acid, but extraordinarily lower ones against acetic acid than against glucose. Accordingly, these organisms aquire the metabolic activities against free acids more actively, if they grow in the

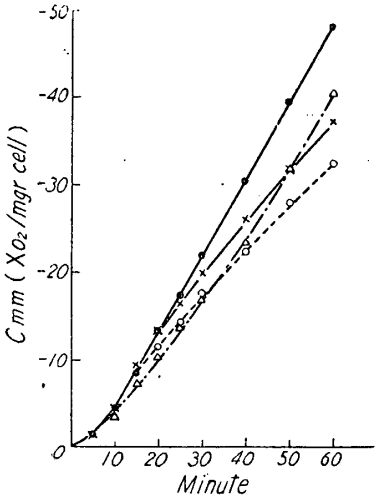


Fig. 5. Oxygen uptake against the acids and glucose by *Strain No. 32*; (●——●) glucose, (×——×) lactic acid, (Δ---Δ) acetic acid, (O·····O) sodium lactate; that measured at 37°, pH 3.5 (phosphate); V_F 3.5 ml.; and molarity of eaeh substrate solution is M/35.

Organisms have been cultured at 30° for 42 hours in medium I with shaking.

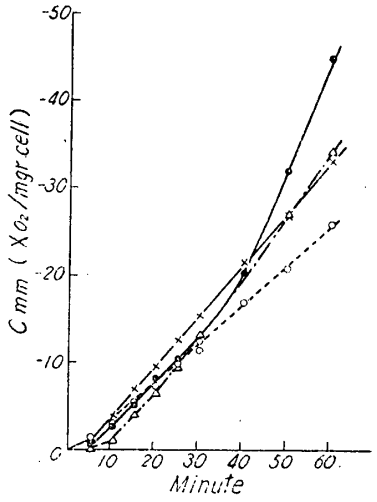
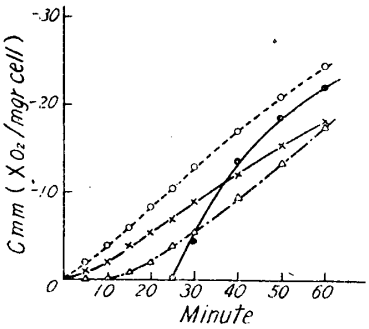


Fig. 6. Oxygen uptake against the acids and glucose by *Strain No. 39*; (●——●) glucose; (×——×) lactic acid, (Δ---Δ) acetic acid, (O·····O) sodium lactate; that measured at 37°, pH 3.5 (phosphate); V_F 3.5 ml.; and molarity of each substrate solution is M/35.

Organisms have been cultured at 30° for 42 hours in medium I with shaking.

Fig. 7. Oxygen uptake against the acids and glucose by *Sake-yeast Kyôkai No. 6*; (●——●) glucose, (×——×) lactic acid, (Δ---Δ) acetic acid, (O·····O) sodium lactate; that measured at 37°, pH 3.5 (phosphate); V_F 3.5 ml; and molarity of each substrate solution is M/35.

Organisms have been cultured at 30° for 42 hours in medium I with shaking.



absence of glucose but in the presence of lactic acid; strain No. 32 became adapted to acetic acid, while the strain No. 39 did so to lactic acid.

The fermentative activities against glucose in aerobic and anaerobic circumstances are observed further. The results are given in Table 4 and Figures 8, 9, and 10. As seen from the Table 3, the RQ-values are as high as 1.5 to 3.1

Table 4. Relationships between respiration and fermentation.

Strain	Q_{O_2}	$Q^{air}_{CO_2}$	$Q^{N_2}_{CO_2}$	R. Q.	$Q^{air}_{CO_2} / Q^{N_2}_{CO_2}$	$-Q_{O_2}+Q^{N_2}_{CO_2}$
No. 32	−54	81	21	1.5	3.8	75
No. 39	−42	132	95	3.1	1.4	137
Kyôkai No. 6	−30	83	51	2.7	1.6	81

Respiration and fermentation in M/35 glucose at 37° and pH 3.5 (phosphate). Organisms have been cultured at 30° for 40 hours in medium I with shaking.

Strain No. 32 shows considerably low $Q^{N_2}_{CO_2}$ (anaerobic fermentative ability), but its value of Q_{O_2} exceeds twoholds that of $Q^{N_2}_{CO_2}$. On the other hand, strain No. 39 and Sake-yeast show several times higher value of $Q^{N_2}_{CO_2}$ than that of strain No. 32, whereupon these values exceed about twoholds those of Q_{O_2}. The ratio of the aerobic fermentative ability to the anaerobic one is shown in column 6, where the strain No. 32 shows a characteristically high value. Thus, it might be concluded that the strain No. 32, film-former, is more aerophilic than the others. Also noteworthy is the fact that aerobic respiration and anaerobic fermentation occur additively in such yeast strains even under aerobic and strongly acid conditions. This can be seen from the good agreement in the values of column 3 and 1, when the respiratory coefficient is assumed as 1.0. This means that

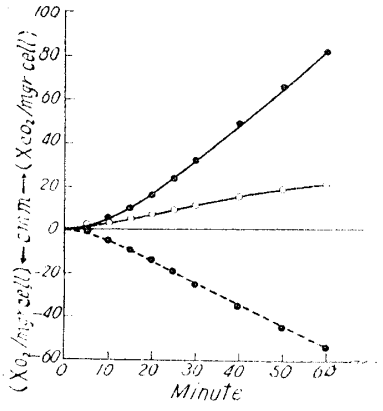


Fig. 8. Output of carbon dioxide in N_2 or air and uptake of oxygen against glucose by *Strain No. 32*; in air (●——●), in N_2 (○——○), and uptake of oxygen (● ····· ●); at 37°; pH 3.5 (phosphate); and molarity of the substrste is M/35. Organisms have been cultured at 30° for 42 hours in medium I with shaking.

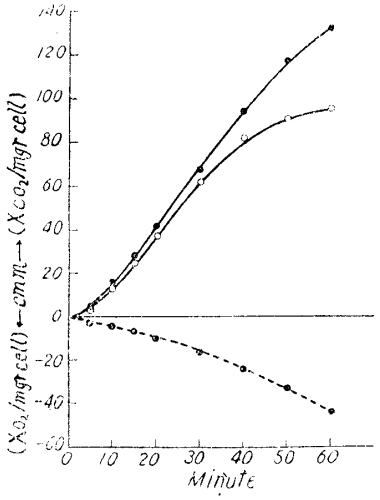


Fig. 9. Output of carbon dioxide in N_2 or air and uptake of oxygen against glucose by *Strain No. 39*; in air (● ——●), in N_2 (○——○) and uptake of oxygen (● ·····●); at 37°; pH 3.5 (phosphate); and molarity of the substrate is M/35. Organisms have been cultured at 30° for 42 hours in medium I with shaking.

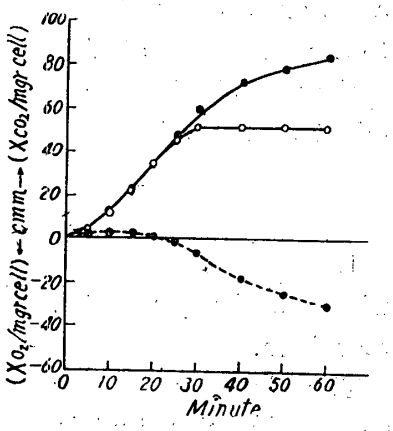


Fig. 10. Output of carbon dioxide in N_2 or air and uptake of oxygen against glucose by *Sake-yeast Kyôkai No. 6* ; in air (●——●), in N_2 (O——O), and uptake of oxygen (●·····●) ; at 37° ; pH 3.5 (phosphate) ; and molarity of the substrate is M/35. Organisms have been cultured at 30° for 42 hours in medium I with shaking.

the fermentative ability of those organisms including Sake-yeast is fairly stable under such conditions.

The above given results were obtained along with the mean velocity of the gas metabolism and it might be true in appearance. In reality the relationship between fermentation and respiration is distinctly competitive as seen in Figures 9 and 10, the gas metabolism is traced with the time. The Sake-yeast shows a typical statue of this competition (Fig. 10). In this case, aerobic fermentation proceeds quite preferably in the early stage by ca. 30. min., and then it suddenly drops down. After this stage the respiratory system replaces the fermentation system, and correspondingly, the oxygen uptake occurs. This competition is observed quite obviously in the case of the strain No. 39. On the other hand, the strain N0. 32, which is well distinguished in its aerophilic character from the other, shows this competitive tendency faintly, so that its aerophilic character is easily discernible (Fig. 8). Consequently, the fermentation system of the less aerophilic yeast is very active but rather unstable especially in acid environment, while this system of the more aerophilic yeast is moderately active but quite stable. The basal mechanisms concerning this phenomena in yeast metablism are an important problem for the future.

At any rate, the ensilage yeast strains show a singnificant metabolic activity against the acids even in the strongly acid environments, and accordingly, it might be concluded that in the phases, probably later than the phase II of ensiling process, where the sugars disappear and instead, the acids are accumulated, the yeasts metabolyze the acids as a carbon source instead of the glucose, and soon or later, make up the neutral environments, which facilitate the growth of the putrefactive aerobes. Thereupon among these yeasts, an aerophilic film-former such as the strain No. 32 may attack preferably the acetic acid, while a less aerophilic non-film-former such as the strain No. 39, chiefly lactic acid. Particularly, the latter yeast displays a useful role to the ensiling process, but reversely, if the aerobic circumstances are more or less established and the

acids are accumulated through the disappearance of the sugars, it turns itself to a harmful role at above cited. It is well known that the yeast is a cause of spoilage of the fermented vegetable food such as sauer kraut or pickels. This might be well adopted in the case of the ensilage.

Summary

1. Green maize ensiling process in the simple earth-pit silo was traced along with the changes in the constituents and the micro-organisms that emerged therefrom.

The whole process was distinguished into four phases, mainly based upon the changes in acids, that is, the phase of acid forming, of flactuation in acids, of equilibrium in acids and of decrease in acids. The causes of these changes are discussed mainly in respect to the actions of micro-organisms.

2. The classification of the 80 strains of the aerobic micro-organisms isolated from the ensilage was based upon their physiological properties. They are divided into sixteen groups by their demand of the carbon sources, organic nitrogen sources and several vitamins from various origins.

These strains were tested as to their tolerance to the strongly acid circumstances. Five strains tolerable to such low pH as 2.4 were obtained and they are determined to be yeasts.

3. Two strains as the representatives of the acid-tolerable yeast above mentioned were studied with regard to their respiratory and fermentative activities against such free acids as lactic and acetic acid by the Warburg's manometric technique, while Kyôkai No. 6, the most typical of Sake-yeast was used as the control.

It was shown that, when grown in an ordinary medium, they could attack both lactic and acetic acid as actively as glucose. When cultivated in a lactate containing medium their activity against the acids accelerated greatly and exceeded that against glucose.

4. The fermentative activities against glucose in aerobic or anaerobic circumstances were studied.

The relationships between fermentation and respiration was observed to be distinctly competitive in the aerobic and strong acid circumstances. When the mean velocity was adopted, it seemed that the aerobic respiration and the anaerobic fermentation occurred additively.

It was also mentioned that the fermentation system of the less aerophilic yeast is very active but rather unstable, especially in acid environments, while this reverse in the case of the more aerophilic yeast.

From the results obtained it was concluded that in ensiled pile the acid tolerable yeasts consumed the acid once accumulated, and made up a circum-

stance, which lead to the putrefaction caused by aerobic bacteria.

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